6.5 significantly ($p<0.05$) reduced filament velocity by 47 and 95%, respectively. In addition, filament velocity was depressed at any given submaximal Ca^{2+} level with the effect significantly more pronounced at pH 6.5 vs 6.8. However, the pCa_{50} increased significantly ($p<0.05$) when the pH was reduced from 7.4 to 6.8 (6.76 ± 0.07 vs. 7.62 ± 0.14) but was not significantly affected at pH 6.5 (6.62 ± 0.82). This suggests a complex relationship between pH and Ca^{2+}-sensitivity. The addition of P_i had no effect on filament velocity at pH 7.4 but significantly reversed the acidosis-induced depression in velocity at both pH 6.8 and 6.5, at every level of Ca^{2+} from pCa 7.0 to pCa 5.0. The addition of P_i also increased the pCa_{50} at pH 6.5 from 6.62 ± 0.82 to 7.74 ± 0.20. The effects of these ions on regulated thin filament velocity are significantly more pronounced than those observed with unregulated actin filaments (Debold et al., 2011); therefore, we are now attempting to identify the structural regions responsible by assessing the functional impact of structural alterations to key regions of Tn.

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Maximum Activation of Thin Filaments at High Ionic Strength requires Bound Calcium and Rigor Myosin

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We have used stopped-flow fluorescence to measure the acceleration of dissociation of hydrolysis products, Pi and deoxymantADP (mdADP), from myosinS1-mdADP-Pi and myosinS1-mdADP by native cardiac thin filaments (TF) at near physiological ionic strength (0.1M KAc). Although the rate constants of the product dissociation steps cannot be measured at this ionic strength, there is a linear dependence of the k_{obs} of product (Pr = mdADP or mdADP-Pi) dissociation (TF + M-Pr ↔ TF-M-Pr → TF-M + Pr) on [TF], which can be used to determine the dependence of the apparent second order rate constant upon the ligands calcium and rigor myosin. The second order rate constant of the dependence of the k_{obs} of Pi dissociation from myosinS1-mdADP-Pi on thin filament (actin subunit) concentration is: $2x10^3$ $M^{-1}s^{-1}$ (no rigor myosin, pCa>8), $5.9x10^4$ $M^{-1}s^{-1}$ (rigor myosin, pCa>8), $1.3x10^5$ $M^{-1}s^{-1}$ (no rigor myosin, pCa<4), and $1.8x10^5$ $M^{-1}s^{-1}$ (rigor myosin, pCa<4). The second order rate constant of the dependence of the k_{obs} of mdADP dissociation from myosinS1-mADP on thin filament concentration is: $4.2x10^4$ $M^{-1}s^{-1}$ (no rigor myosin, pCa>8), $2.3x10^5$ $M^{-1}s^{-1}$ (rigor myosin, pCa>8), $6.2x10^5$ $M^{-1}s^{-1}$ (no rigor myosin, pCa<4), and $1.2x10^6$ $M^{-1}s^{-1}$ (rigor myosin, pCa<4). Thus, neither calcium nor rigor myosin is sufficient to maximally activate thin filament acceleration of product dissociation on its own. Full activation requires both calcium and rigor myosin to be bound to the thin filament. These results are (i) similar to data previously obtained at low ionic strength; (ii) inconsistent with models which predict that rigor myosin fully activates the thin filament and (iii) support a mechanism of thin filament regulation in which the principal regulated step of the hydrolysis cycle is phosphate dissociation from actomyosin-ADP-Pi.

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S-Nitrosylation Decreases Ca^{2+} Sensitivity and Actomyosin ATPase Activity of Contractile Proteins in Cardiac Myofibrils

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Increases in muscle nitric-oxide (NO) production can be buffered by reaction with intracellular glutathione, forming S-nitrosoglutathione (GSNO). GSNO has been shown to S-nitrosylate Cys thiols of cardiac contractile proteins in vivo and in vitro, but effects on maximal force, thin-filament Ca^{2+} sensitivity and actomyosin ATPase activity are unknown. Here, we analyzed the targets of S-nitrosylation in mouse cardiac contractile proteins, and examined the effects of these modifications on function in myocytes and skinned cardiac myofibrils. S-Nitrosylation and denitrosylation were detected using resin-assisted capture (SNO-RAC) and targets for S-nitrosylation were identified by quantitative LC-MS/MS. Isolated cardiomyocytes treated with S-nitrosocysteine (CysNO, 500μM, 10min) showed an increase in total protein-SNO, followed by progressive denitrosylation (30-60min with CysNO). At 10min, CysNO dose-dependently increased S-nitrosylation of specific Cys thiols in myosin heavy chain, actin, TnC, TnI, myosin-binding protein C and other muscle proteins. Myofibril thin-filament Ca^{2+} sensitivity decreased ($P<0.05$) after in-vitro treatment with pharmacological GSNO concentrations (1, 10, 100 μM), but maximum force did not change ($P>0.05$). Loss of Ca^{2+} sensitivity was partially reversed by the denitrosation agent, ascorbate. Relaxation kinetics of skinned fibers, as measured by flash photolysis, were also significantly reduced by100μM GSNO (k1,15.33 to 11.68/s; k2 2.33 to 0.87/s; fit to double exponential). Maximal myofibrillar ATPase activity (pCa 5.0) was also dose-dependently inhibited (8, 15, 30%) by 50, 100 and 500μM GSNO, an effect that was reversed by ascorbate. The findings suggest that S-nitrosylation of regulatory Cys thiol(s) can reversibly modulate cardiac muscle contraction and may be able to protect the heart by reducing myosin ATPase demand without affecting the maximal force of contraction.

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A Novel Role of Caldesmon during Smooth Muscle Relaxation

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The effect of h-caldesmon (h-CaD) on smooth muscle contractility was examined by force measurements on tissues isolated from wild-type (WT) and h-CaD-null (KO) mice. Both aorta and tail arteries from KO mice relaxed more slowly than that from WT animals. The force decay of beta-escin-permeabilized, kinase-inhibited tissues under pCa>9 was best fitted with a two-exponential process. We found that the apparently slower relaxation for the KO samples was not due to lower rate constants, but rather, was because of increased amplitude of the slower component of the decay. To search for an explanation for the observed relaxation kinetics, we drew some lessons from the work done on striated muscles. Muscle relaxation involves two major events: crossbridge detachment and change of the thin filament from active to inactive state. The latter occurs first, corresponding to the initial lag phase, followed by the rapid force decay, the crossbridge detachment. In smooth muscle, the lag phase is ascribed to myosin dephosphorylation, but it may also include the filament state change, i.e., CaD-tropomyosin moving to a position on actin filaments that hinders myosin binding. This ''off-position'' is under strain, because the dephosphorylated crossbridges are still attached. Assuming that there are two possible positions for CaD-tropomyosin to occupy, one displacing the bound myosin more promptly than the other, the crossbridge would detach at two different rates, resulting in the observed biphasic decay. Our data then suggest that when h-CaD is absent, fewer tropomyosin molecules are at the blocking position. Thus, the return of smooth muscle tropomyosin to the proper inhibitory position upon relaxation needs help from h-CaD. In striated muscle the movement of tropomyosin was shown to be facilitated by troponin subunits. CaD may play a troponin-like role in smooth muscle both structurally and functionally.

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Stable Double-Headed Structure is Essential not only for the Inhibited State, but also for the Fully Activated State of Smooth Muscle Myosin

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It is generally accepted that dephosphorylation of regulatory light chain (RLC) induces the interactions between the two heads of smooth muscle myosin (Sm) and between the head and the tail, thus inhibiting the motor activity, and phosphorylation of RLC interrupts above interactions, thus reversing the inhibition and stimulating the motor activity to maximal value. Thus it is predicted that Sm subfragment-1 (S1) containing only one head without the tail is fully active. However, no solid evident so far supports this prediction, although early studies showed that S1 produced by limited protease treatment is partially active. Here we produced a number of Sm truncations with various length of the tail. The stability of double-headed structure of Sm is dependent on the length of the coiled-coil tail. The Sm truncations with coiled-coil longer than 214 amino acids form stable double-headed structure (stable HMM), those with coiled-coil shorter than 179 amino acids form unstable double headed structure (unstable HMM), and that without coiled-coil, i.e. S1, is completely single-headed. Phosphorylation of RLC regulates the motor activity of stable HMM completely, regulates that of unstable HMM partially, and does not regulate that of S1. Unexpectedly, the actin-activated ATPase activity of S1, either unphosphorylated or phosphorylated, is higher than that of unphosphorylated stable HMM but less than 10% of that of phosphorylated stable HMM. The actin-activated ATPase activities of unphosphorylated Sm truncations increase with the shortening of the coiled-coil tail, and that of phosphorylated Sm truncations decrease with the shortening of the coiled-coil tail. These results indicate that the stable double-headed structure is critical not only for the inhibited